



## Protective Activity of Citicoline Against Neurobehavioral and Cerebellar Histo-biochemical Derangement Induced by Nickel Chloride: Role of Caspase-3 and NRF2

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### Abstract

**Context:** Nickel chloride ( $\text{NiCl}_2$ ) is a widely used industrial compound that poses significant neurotoxic risks through mechanisms involving oxidative stress, apoptosis, and neuronal degeneration. The cerebellum is particularly susceptible to such insults due to its role in motor coordination and locomotor control.

**Objective:** The study investigated the neuroprotective potential of citicoline against  $\text{NiCl}_2$ -induced cerebellar toxicity in adult Wistar rats.

**Materials and Methods:** Forty-two adult Wistar rats were randomly assigned to six groups and treated for 28 days as follows: I (Control), II (5mg/kg  $\text{NiCl}_2$ ), III (250 mg/kg citicoline + 5mg/kg  $\text{NiCl}_2$ ), IV (500 mg/kg citicoline + 5mg/kg  $\text{NiCl}_2$ ), V (250 mg/kg citicoline) and VI (250 mg/kg citicoline). Neurobehavioural assessments were performed using the Open Field Test, String Test, and Movement Initiation Test. Oxidative stress markers, including malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and reduced glutathione (GSH), were evaluated in cerebellar homogenates. Histological examination of the cerebellum was conducted, while molecular docking studies were performed to investigate the interaction of citicoline with Caspase-3 and nuclear factor erythroid 2-related factor 2 (NRF2).

**Results:** Compared to control,  $\text{NiCl}_2$  significantly ( $p < 0.05$ ) impaired locomotor and motor performance and induced oxidative stress (decreased SOD, CAT, GPx, and GSH; increased MDA), alongside histological evidence of Purkinje cell degeneration, nuclear pyknosis, and cerebellar layer vacuolations. Citicoline significantly ( $p < 0.05$ ) reversed these behavioural, biochemical, and histological alterations when compared to rats treated with  $\text{NiCl}_2$  only, and showed favourable docking affinities towards Caspase-3 and NRF2.

**Conclusion:** Citicoline protects against  $\text{NiCl}_2$ -induced cerebellar toxicity by attenuating oxidative stress and possibly modulating apoptotic pathways, thus preserving neuronal integrity and highlighting its therapeutic potential for heavy metal-induced neurodegeneration and motor dysfunction.

Keywords: Citicoline; Nickel chloride; Cerebellum; Caspase-3; NRF2

### Introduction

Numerous studies have shown that prolonged or excessive exposure to metals leads to their accumulation

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in the soft tissues of the body<sup>1</sup>. Although some metals, including iron, zinc, copper, chromium, and manganese, are essential for normal physiological processes in trace amounts, elevated concentrations can result in toxic effects. Such toxicity occurs when these metals aberrantly bind to protein sites, displacing essential ions from their physiological

positions and consequently disrupting normal cellular functions<sup>2</sup>. In addition, metals can induce oxidative damage to key biomolecules, including carbohydrates, proteins, and nucleic acids, primarily through interactions with DNA and nuclear proteins<sup>3</sup>. These disruptions may ultimately impair vital functions, manifesting as deficits in motor, sensory, emotional, and cognitive activities<sup>4,5</sup>.

Among toxic metals, nickel (Ni) is of considerable concern due to its extensive industrial applications and environmental persistence. Its widespread use in electroplating, battery production, and alloy manufacturing has led to increased environmental dissemination and human exposure. Nickel toxicity affects multiple organ systems; however, the central nervous system is particularly vulnerable<sup>6,7</sup>. Exposure to nickel occurs through occupational, environmental, and dietary sources, including industrial emissions, contaminated water, and food<sup>6,8</sup>. Neurotoxic effects of nickel exposure have been associated with oxidative stress, neuroinflammation, and neuronal degeneration, leading to deficits in motor coordination, locomotor activity, and overall cerebellar function<sup>9,10</sup>. Such impairments may manifest as altered motor performance, reduced coordination, and disturbances in balance, highlighting the cerebellum as a critical target of nickel-induced toxicity.

In the search for effective and safer therapeutic interventions against heavy metal-induced neurotoxicity, attention has shifted towards neuroprotective agents with antioxidant and cytoprotective properties. Citicoline (cytidine-5'-diphosphocholine), an endogenous intermediate in phospholipid metabolism, has gained recognition as a promising therapeutic candidate due to its broad neuroprotective profile<sup>11</sup>. It contributes to the preservation of neuronal membrane integrity, promotes phosphatidylcholine biosynthesis, and supports neurotransmission, particularly within cholinergic systems<sup>12</sup>. In addition, citicoline exerts significant antioxidant effects by attenuating reactive oxygen species, enhancing glutathione levels, and preserving mitochondrial function<sup>11,13</sup>. Moreover, citicoline has been reported to possess anti-inflammatory, anti-apoptotic, and neurorestorative effects, contributing to improved

neuronal survival and functional recovery<sup>14,15,16</sup>. Although the neuroprotective activity of citicoline has been previously reported, its potential protective effects against nickel-induced cerebellar toxicity remain poorly explored. Accordingly, this study aimed to investigate the neuroprotective potential of citicoline in the cerebellum of Wistar rats exposed to Nickel chloride.

## Materials and methods

### Animals and Treatment Regimen

Forty-two male Wistar rats (170–190 g) were used for this study. The animals were randomly assigned to six groups (n = 7 per group). The animals were housed in polypropylene cages under standard laboratory conditions at room temperature, with free access to food and water and were allowed to acclimatize for 14 days. All experimental procedures were conducted in accordance with international guidelines for the care and use of laboratory animals. Ethical approval was obtained from the Research Ethics Committee, College of Medical Sciences, University of Benin, Benin City, Nigeria, with REC Approval Number: CMS/REC/2024/662. The experimental design is shown in Table 1.

**Table 1: Experimental design**

| GROUPS                               | TREATMENT                                        |
|--------------------------------------|--------------------------------------------------|
| Group I (Control)                    | 1 mL distilled water                             |
| Group II (NiCl <sub>2</sub> )        | 5mg/kg Nickel chloride (NiCl <sub>2</sub> ) only |
| Group III (CT1 + NiCl <sub>2</sub> ) | 250 mg/kg citicoline + 5mg/kg NiCl <sub>2</sub>  |
| Group IV (CT2 + NiCl <sub>2</sub> )  | 500 mg/kg citicoline + 5mg/kg NiCl <sub>2</sub>  |
| Group V (CT1)                        | 250 mg/kg citicoline                             |
| Group VI (CT2)                       | 500 mg/kg citicoline                             |

Oral citicoline administrations were delivered one hour before intraperitoneal administration of NiCl<sub>2</sub> daily, for twenty-eight days.

### Neurobehavioural Assessments

**Open Field Test (OFT):** OFT was performed as earlier reported by our laboratory in a 72 cm × 72 cm × 20 cm wooden open box with lines on its floor dividing it into 18 cm by 18 cm<sup>17,18</sup>. Parameters such as rearing, grooming, ambulation, immobility, thigmotaxis, sniffing, line crossing, and central square entry were evaluated.

**String Test:** This is commonly utilized to assess grip

strength<sup>19</sup>. Here, rats used their forepaws to hold a 2 mm diameter x 60 cm length x 50 cm height steel wire. Latency to grip loss was recorded as the duration of time spent (maximum 180 seconds) till it fell<sup>20</sup>.

**Movement Initiation Test:** The Movement Initiation test (MIT), often utilized to evaluate motor function, was performed as previously described<sup>19,21</sup>. The time taken for the initiation of one step was recorded for each forelimb and averaged to attain the movement initiation score.

### Oxidative Stress Assessments

Following sacrifice under light chloroform anaesthesia and cervical dislocation, brains were excised and the cerebellum isolated for analysis. Cerebellar tissues were rinsed in cold phosphate-buffered saline (PBS), homogenized in ice-cold 20 mM Tris-HCl buffer (pH 7.4), and centrifuged at  $10,000 \times g$  for 10 minutes at 4 °C, as earlier reported<sup>21,22</sup>. The supernatant was collected for the assessment of oxidative stress markers, including malondialdehyde (MDA)<sup>23</sup>, catalase (CAT)<sup>24</sup>, superoxide dismutase (SOD)<sup>25</sup>, glutathione peroxidase (GPx)<sup>26</sup>, and glutathione (GSH)<sup>27</sup>.

### Histological Assessments

The harvested cerebella were fixed for 72 hours in 10% buffered formal saline. The Haematoxylin and Eosin staining methods were utilized as the processing protocol, as earlier reported<sup>28</sup>.

### Molecular Docking Study

In silico molecular docking of citicoline was performed against Caspase-3 and Nuclear factor erythroid 2-related factor 2 (NRF2). The crystal structures of Caspase-3 and NRF2 were retrieved from the Protein Data Bank. Docking simulations were carried out using AutoDock Vina as previously described<sup>29</sup>, and binding affinities were expressed in kcal/mol. Protein-ligand interactions were visualized using BIOVIA Discovery Studio 2019 for 2D representations and PyMOL Molecular Graphics Software for 3D surface views, as

previously reported<sup>30</sup>.

## Results

### Effect of Treatment on Neurobehaviour

Figure 1 shows the effect of treatment on open field parameters across the experimental groups. Rearing, ambulation, line crossing and central square entry (CSE) showed a significant decrease ( $p < 0.05$ ) in NiCl<sub>2</sub>-only treated rats compared to the control group. However, pre-treatment with citicoline significantly increased ( $p < 0.05$ ) these neurobehavioural parameters when compared to the NiCl<sub>2</sub>-only group. Also, grooming, immobility, thigmotaxis and sniffing showed a significant increase ( $p < 0.05$ ) in NiCl<sub>2</sub>-only exposed rats when compared to the control group. However, pre-treatment with citicoline displayed a significant reduction ( $p < 0.05$ ) in these parameters when compared to the NiCl<sub>2</sub>-only group.

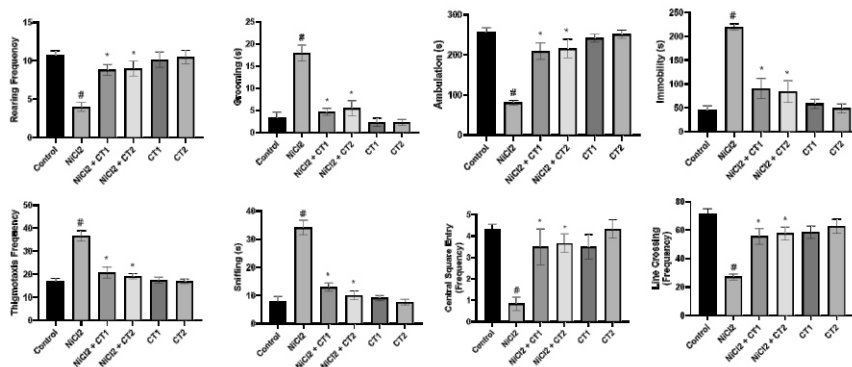


**Figure 1:** Open field test parameters across experimental groups.

#  $p < 0.05$  compared with the control group; \*  $p < 0.05$  compared with the NiCl<sub>2</sub>-only group.

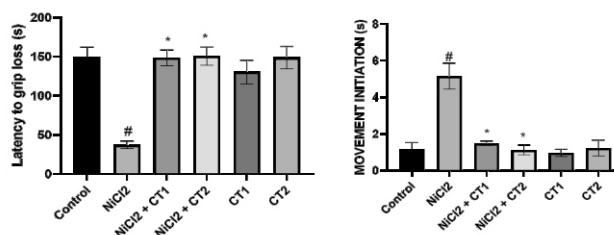


**Figure 2:** Latency to grip loss and movement initiation tests across experimental groups.

#  $p < 0.05$  compared with the control group; \*  $p < 0.05$  compared with the NiCl<sub>2</sub>-only group

Figure 2 shows the effect of treatment on latency to grip loss and movement initiation across the

experimental groups. A significant decrease ( $p < 0.05$ ) and a concurrent significant increase ( $p < 0.05$ ) were observed in the  $\text{NiCl}_2$ -only treated rats when compared to control. However, a significant increase ( $p < 0.05$ ) in latency to grip loss and a corresponding significant decrease ( $p < 0.05$ ) in movement initiation were observed in the  $\text{NiCl}_2$ -exposed rats pre-treated with citicoline when compared to the  $\text{NiCl}_2$ -only group.

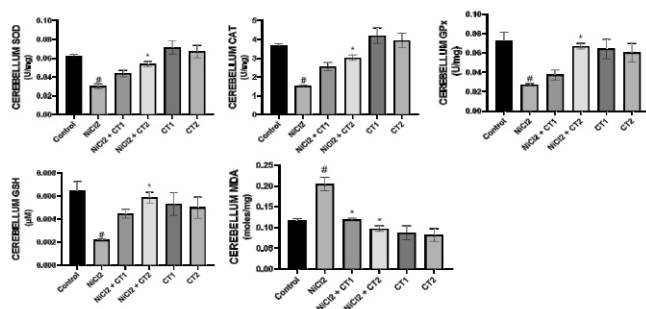


Figure 3: Antioxidant and Lipid peroxidation findings across experimental groups.  
# $p < 0.05$  compared with the control group \* $p < 0.05$  compared with the  $\text{NiCl}_2$ -only group

### Effect of Treatment on Oxidative Stress

Figure 3 shows the effect of treatment on oxidative stress parameters. A significant reduction ( $p < 0.05$ ) in the activities of antioxidant enzymes (SOD, CAT, GPx, GSH), and a significant increase ( $p < 0.05$ ) in lipid peroxidation (MDA) were observed in the  $\text{NiCl}_2$ -only treated rats when compared to control. However, a significant increase ( $p < 0.05$ ) in SOD, CAT, GPx and GSH and a significant decrease ( $p < 0.05$ ) in MDA concentration were noticed in the  $\text{NiCl}_2$ -exposed rats treated with citicoline ( $\text{NiCl}_2 + \text{CT1}$  and  $\text{NiCl}_2 + \text{CT2}$ ) when compared to the  $\text{NiCl}_2$ -only treated rats.

### Effect of Treatment on Histology

Plate 1 (A–F) shows the representative histology of the cerebellum in control and treatment groups. Plate 1A revealed normal structure of the cerebellum layers (molecular, Purkinje cell and granular cell layers). Plate 1B ( $\text{NiCl}_2$ -only) showed degenerating Purkinje cells (big arrows), with nuclei appearing irregular, darkly stained and pyknotic. Also observed are vacuolations in the molecular, Purkinje and granular cell layers. Plates

1C ( $\text{CT1} + \text{NiCl}_2$ ) and 1D ( $\text{CT2} + \text{NiCl}_2$ ) showed relatively normal histological structure of cerebellar layers observed. Plates 1E (CT1) and 1F (CT2) groups showed relatively normal cerebellar histological layers.

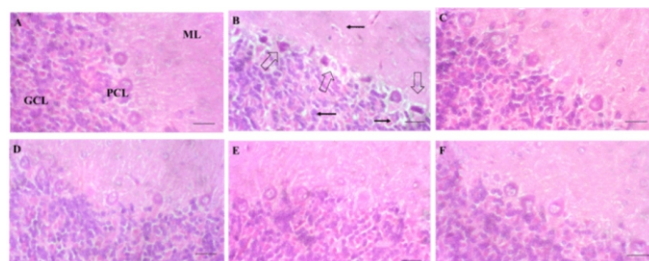


Plate 1: Representative histology of the cerebellum in control and treatment rats. (A) Normal histological structure of cerebellum layers – Molecular layer (ML); Purkinje Cell layer (PCL); Granular Cell layer (GCL); (B) cerebellum showing degenerating Purkinje cells (big arrows), with nuclei appearing irregular, darkly stained and pyknotic. Also observed are vacuolations in the Molecular, Purkinje and granular cell layers (arrows). (C–F) Relatively normal histological structure of cerebellum layers observed. (H&E; Scale bar: 25 $\mu\text{m}$ )

Table 2: Binding affinities of Citicoline and reference compounds against Caspase-3 and NRF2

| Compound             | Caspase-3 (kcal/mol) | Nrf2 (kcal/mol) |
|----------------------|----------------------|-----------------|
| Citicoline           | -6.7                 | -5.4            |
| Levodopa (Standard)  | -5.3                 | -5.8            |
| Vitamin E (Standard) | -6.1                 | -5.8            |

### In Silico Findings

The molecular docking results of citicoline and the reference compounds against Caspase-3 and nuclear factor erythroid 2–related factor 2 (NRF2) are presented in Table 2. Citicoline exhibited binding energies of  $-6.7$  kcal/mol and  $-5.4$  kcal/mol towards Caspase-3 and NRF2, respectively. Against Caspase-3, citicoline demonstrated a stronger binding affinity than levodopa ( $-5.3$  kcal/mol) and vitamin E ( $-6.1$  kcal/mol), indicating favourable interaction with this key apoptotic mediator. In contrast, citicoline showed a slightly lower binding affinity towards NRF2 compared with Levodopa

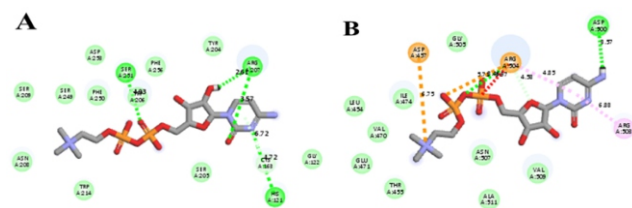


**Figure 4:** 2D surface view of citicoline at the active site of (A) Caspase-3 (B) nuclear factor erythroid 2-related factor 2 (Nrf-2)

and Vitamin E, both of which recorded binding energies of  $-5.8$  kcal/mol.

The two-dimensional (2D) ligand-protein interaction maps of citicoline within the active sites of caspase-3 and Nrf2 are presented in Figures 4A and 4B, respectively. The interaction profiles reveal the specific amino acid residues involved in binding and illustrate the molecular basis underlying the observed docking affinities.

## Discussion

Nickel chloride ( $\text{NiCl}_2$ ) is a neurotoxic heavy metal that induces oxidative stress, apoptosis, and neuronal damage<sup>31,32</sup>. This study investigated the neuroprotective effects of citicoline against  $\text{NiCl}_2$ -induced cerebellar toxicity.

Behavioural assessment remains one of the most sensitive approaches for detecting neurotoxicity, as alterations in locomotor and exploratory activities often precede overt structural damage. In the present study,  $\text{NiCl}_2$  exposure significantly reduced rearing frequency, ambulation, line crossing, and central square entry in the open field test, indicating impaired locomotor activity, reduced exploratory drive, and compromised behavioural adaptability. These findings are consistent with previous reports showing that nickel accumulation within neural tissues disrupts neurotransmitter homeostasis and neuronal signalling pathways involved in motor coordination and behavioural regulation<sup>31,33</sup>. Conversely, rats pre-treated with citicoline demonstrated significant improvement in these behavioural parameters, suggesting preservation of neuronal function and restoration of normal locomotor activity.

$\text{NiCl}_2$  exposure also increased grooming, immobility, thigmotaxis, and sniffing behaviours, further indicating impaired motor performance and

altered exploratory behaviour. Increased immobility reflects reduced spontaneous movement and behavioural responsiveness<sup>34</sup>, whereas elevated thigmotaxis may indicate reluctance to explore unfamiliar environments<sup>35</sup>. Citicoline significantly attenuated these abnormalities, demonstrating its ability to improve locomotor performance and behavioural responsiveness. These beneficial effects may be related to its capacity to preserve neuronal membrane integrity, enhance cholinergic neurotransmission, and support neuronal repair mechanisms<sup>11</sup>. Similar observations were obtained in the string test and movement initiation test, where  $\text{NiCl}_2$ -treated rats exhibited reduced latency to grip loss and prolonged movement initiation time, indicative of impaired neuromuscular strength, motor coordination, and motor planning. The significant improvement observed following citicoline treatment further supports its role in maintaining cerebellar function and motor pathway integrity.

Oxidative stress is widely recognized as a major mechanism underlying heavy metal-induced neurotoxicity<sup>36-38</sup>. Nickel exposure promotes excessive generation of reactive oxygen species (ROS), resulting in oxidative damage to cellular macromolecules and disruption of endogenous antioxidant defence systems. In the present study,  $\text{NiCl}_2$  exposure significantly reduced the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and reduced glutathione (GSH) while markedly increasing malondialdehyde (MDA) levels. These findings indicate substantial oxidative stress and enhanced lipid peroxidation within cerebellar tissue. Similar observations have been reported in previous studies demonstrating that nickel-induced neurotoxicity is mediated through ROS overproduction, mitochondrial dysfunction, and oxidative damage to neuronal membranes<sup>19,39,40</sup>. The increase in MDA further confirms membrane lipid peroxidation, a major consequence of oxidative injury in neural tissues. Because the brain possesses high oxygen demand and abundant lipid content, it is particularly susceptible to free radical-mediated damage. Notably, citicoline significantly restored antioxidant enzyme activities and reduced MDA concentrations compared with the  $\text{NiCl}_2$ -only group. These findings suggest that citicoline

effectively attenuated oxidative stress and preserved redox homeostasis. The antioxidant effects of citicoline may be attributed to its ability to stabilize mitochondrial membranes, enhance phospholipid synthesis, improve cellular energy metabolism, and reduce ROS generation. By preserving antioxidant capacity and limiting lipid peroxidation, citicoline likely interrupts the cascade of events leading to neuronal degeneration.

The histological findings further corroborated the behavioural and biochemical observations. Histological examination of the cerebellum revealed marked neurodegenerative changes in  $\text{NiCl}_2$ -treated rats, including degeneration of Purkinje cells, nuclear pyknosis, and extensive vacuolations within the molecular, Purkinje, and granular layers. Purkinje cells are the principal output neurons of the cerebellar cortex and are essential for motor coordination and balance<sup>41</sup>. Therefore, degeneration of these neurons is closely associated with impaired cerebellar processing and motor dysfunction. These findings agree with previous reports implicating oxidative stress and apoptosis in nickel-induced neuronal injury<sup>33,40,42</sup>. In contrast, citicoline pre-treatment preserved the structural integrity of the cerebellum, with treated groups exhibiting relatively normal cerebellar architecture and minimal evidence of neuronal degeneration. The preservation of Purkinje cell morphology and cerebellar layers suggests that citicoline effectively protects neural tissue against nickel-induced damage. This neuroprotective effect is likely mediated through attenuation of oxidative stress, stabilization of neuronal membranes, maintenance of mitochondrial function, and modulation of apoptotic pathways.

Apoptosis represents another important mechanism involved in nickel-induced neurotoxicity. Excessive oxidative stress can activate apoptotic pathways, resulting in progressive neuronal loss and functional impairment<sup>43</sup>. To further explore the molecular basis of citicoline-mediated neuroprotection, molecular docking studies were conducted against Caspase-3 and NRF2, two key regulators of apoptosis and antioxidant defence, respectively. Citicoline exhibited a binding energy towards caspase-3 which was stronger than those observed for levodopa and vitamin E. This favourable binding affinity suggests that citicoline may modulate caspase-dependent

apoptotic signalling and thereby reduce neuronal cell death following nickel exposure. Citicoline also demonstrated a stable interaction with NRF2. Although slightly lower than those recorded for levodopa and vitamin E, the interaction suggests a potential role in enhancing antioxidant defence mechanisms through NRF2-related pathways. Furthermore, the two-dimensional ligand-protein interaction maps revealed favourable interactions between citicoline and amino acid residues within the active sites of both proteins, providing molecular support for its antioxidant and anti-apoptotic activities.

### Conclusion

Findings from this study indicate that citicoline attenuates  $\text{NiCl}_2$ -induced cerebellar toxicity primarily through its antioxidant activity, resulting in the preservation of cerebellar histoarchitecture and improvement of locomotor and motor functions. The observed restoration of endogenous antioxidant defences, reduction in lipid peroxidation, and favourable interactions with Caspase-3 and NRF2 further support its neuroprotective potential. Therefore, citicoline may serve as a promising therapeutic candidate for the prevention and management of nickel-induced neurotoxicity and its associated motor dysfunction.

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